# NOTES ON A 32 MILLIMETER FREEMARTIN.

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In an earlier paper, Bissonnette ('24) described the development of the reproductive ducts and canals in cattle embryos from 28.0 mm. crown-rump length to 20.0 cm., and compared with that series one of 5 freemartins from 9.0 cm. to 20.0 cm. In that paper the gonad of a 9.0 cm. freemartin was described which showed unmistakable development of ovarian cortex or secondary sexcord region. The material described gave support to the theory of Lillie ('16 and '17) and Keller and Tandler ('16) that the freemartin is a female modified in utero in the male direction by the action of a male hormone from the testis of her twin brother. It showed that she may go so far in the female direction as to have a secondary sexcord region and even to the condition with an intermittent wolffian duct before undergoing regression of the müllerian duct derivatives and added growth and differentiation of the so-called "male" structures. The one there described was the first showing such sexcords. Since earlier writers had agreed that such cords were never found in freemartins (Chapin, '17, Willier, '21, Lillie, '23) the question remained as to whether this was not a very abnormal situation among freemartins. Further study of younger stages of the freemartin was needed to answer this question.

Lillie ('23) described and figured a 3.75 cm. freemartin with no such cords but with a germinal epithelium more than one cell thick—in some places even 3 or 4 cells thick—but never more than about a third to a sixth of the thickness of the corresponding layer in the single-born female. He found the ducts and canals in no way different from those of the female of corresponding size. This was the smallest stage of the freemartin described and it showed definite modification of the gonad in the male direction.

The subject of the study described in this paper is a freemartin

of 32 mm. crown-rump length which is of interest because (1) it is the smallest stage so far studied, (2) it has a minimum of union of the chorions compatible with any blood transfusion, (3) it is another case where ovarian cortex is found outside the tunica albuginea, (4) it appears to be as near as possible to the stage of distinguishable sex-differentiation in which modifications of the freemartin type can be distinguished, (5) it shows slight free-martin modification and forms the initial end of a series of inter-grades between the postnatal freemartin types and the normal female.

## Material and Methods.

This 32 mm. freemartin, twin to a 34 mm. male, was taken July 28, 1923, at the killing floor of Swift & Co., Chicago Stockyards. Since it was the smallest freemartin in over 50 taken up to that time by the writer, it was especially carefully treated.   The uterus of the mother was slit open throughout both horns and the mem-branes and twins carefully lifted out.   The embryos were far up the opposite horns of the uterus and the chorions were but slightly united and showed no bloodvessel union visible to the naked eye. The placentæ pulled apart with a small amount of bleeding only, when they were handled after removal, though they held together during that operation.  Chorions and embryos entire were placed at once into Bouin's fluid which was changed once about two hours after they were put in.   No injection was attempted since the vessels were judged too small for the apparatus at hand on the killing floor and the help available, and the internal organs were wanted for microscopic work and early fixation was desired. The membranes and embryos were washed in 50 and 70 per cent. alcohols, to the latter of which at its second addition a few drops of a saturated aqueous solution of lithium carbonate was added to help remove the picric acid.   They were then stored in 80 per cent. alcohol for about a year and a half, when the embryos were removed and the posterior parts of both embryos were stained in bulk in alum cochineal and sectioned 30 micra thick. The series was counterstained on the slide in orange G and mounted in balsam.

Both maternal ovaries had been taken by the boy who collects those with large corpora lutea for extraction, so it is supposed each had a corpus luteum.
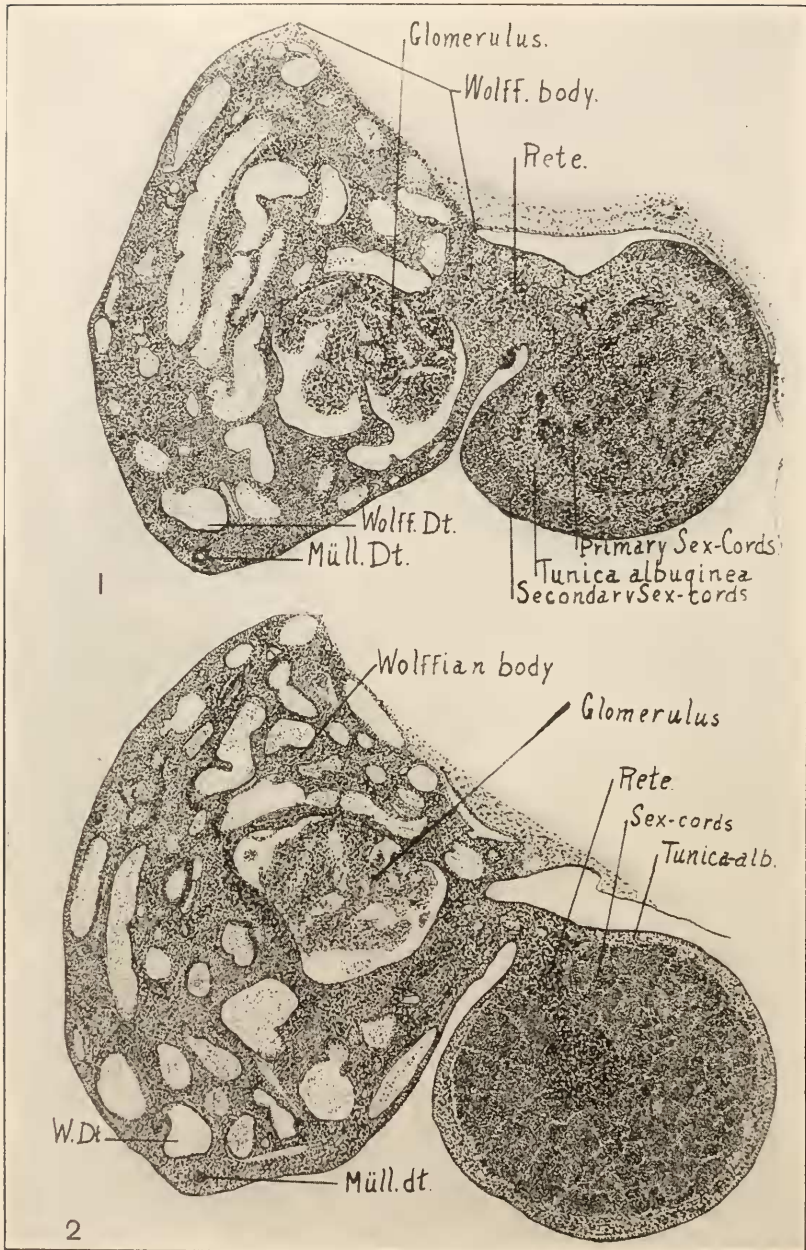
FIG. 1. Transverse section through gonad, mesonephros, and ducts of right side of
normal female, 32 mm. crown-rump length. × 38.3.

FIG. 2. Transverse section through right gonad, mesonephros, and ducts of
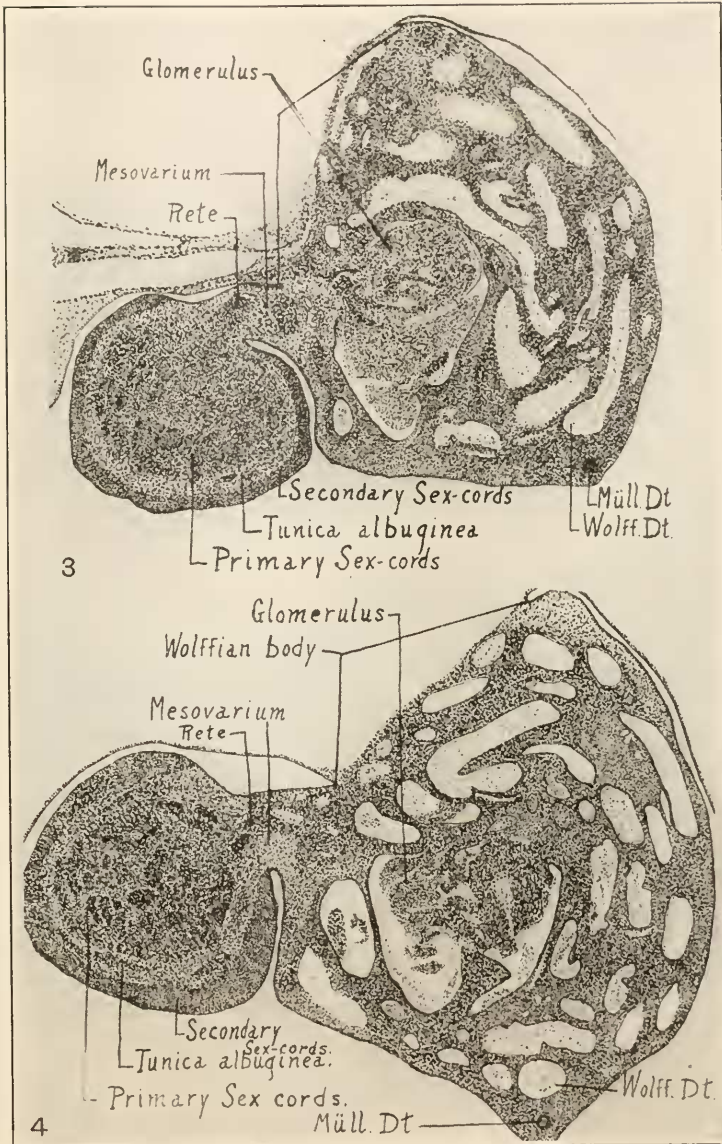normal male, 32 mm. crown-rump length. × 38.3.

FIG. 3. Transverse section through left gonad, mesonephros, and ducts of free-
martin, 32 mm. crown-rump length. × 34.3.

FIG. 4. Transverse section through left gonad, mesonephros, and ducts of normal
female, 32 mm. crown-rump length. × 34.3.

On washing out, no vascular anastomosis could be made out
with the binocular dissecting microscope, low power, but the
bleeding on separation was evidence that there was capillary
connection at least.

Previous work had shown that thick sections have some ad-
vantages over thin in showing the zones in the gonads and regions
of even slight degeneration with consequent pigment production.
They also save time in reconstruction of the ducts and canals.
Such reconstruction was made for this freemartin and for a normal
female of the same size for a control, after the manner of Bis-
sonnette ('24). The notochord was used as reference line for
dorso-ventral relations of ducts, canals, mesonephros, and anlagen
of the round ligament or gubernaculum, and gonads (Figs. 5 and
6). Typical cross-sections through the gonad, mesonephros,
müllerian and wolffian ducts, in the normal female, male, and
freemartin, are shown in Figs. 1, 2, 3, and 4.

### Description and Comparison with Normals.

*The Placental Situation.*—The twins were far apart in discrete
but united chorions with cotyledons beginning to appear as
opaque patches. One chorion was telescoped slightly into the
end of the other and they were so united that bleeding occurred
on separation. Otherwise the chorions were normal. Both
older and younger cases have been taken by the writer in which
the chorions were in contact but not so adherent as to cause
bleeding on separation. In all such cases so far investigated the
female has proved normal. This situation was much the same as
that in the 9.0 cm. freemartin No. 1, Fig. 2, of Bissonnette's ('24)
paper, where a slight though definite amount of modification in
the male direction was demonstrated and secondary sexcord re-
gions or cords of Pflüger were found. It is another case permit-
ting a small blood interchange between the twins and a relatively
small concentration of hormone would be expected on the female
side and so a longer period might elapse before the effective
minimum concentration of the hormone is reached. The am-
nions were separated widely and were normal.

*The External Genitalia.*—Externally this freemartin was female
in type as shown by Figs. 5 and 6 in sagittal section. The

clitoris was slightly more recurved than that of the normal control. However there are variations in this respect among single females due to the difference in relative curvatures of the embryos at the time of killing and fixing. It resembles the one figured
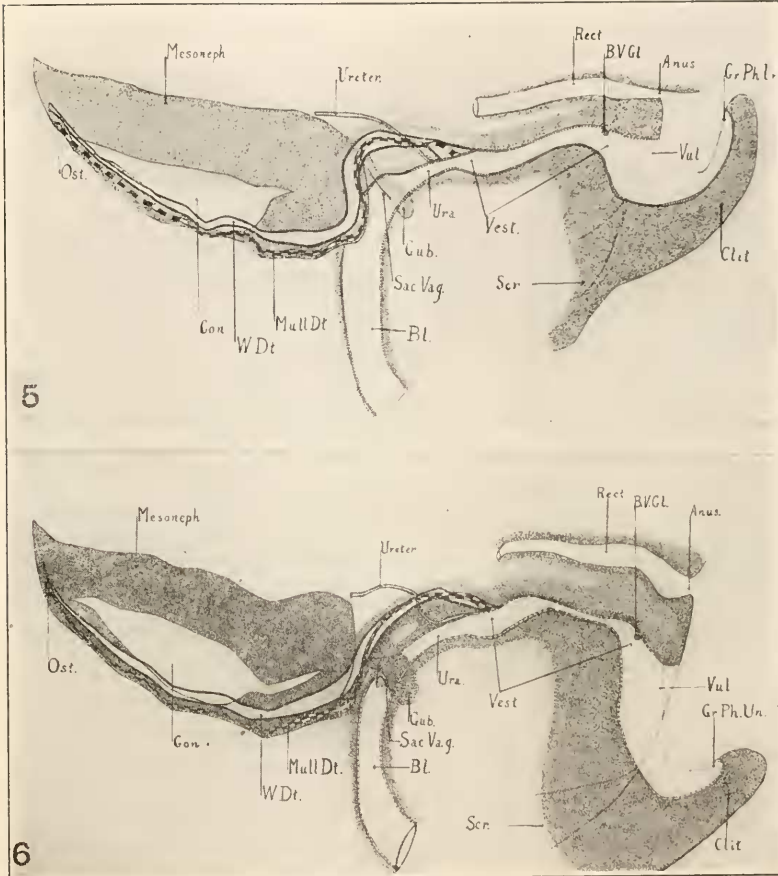


FIG. 5. Reconstruction of the urinogenital system of the freemartin of 32 mm. crown-rump length. × 9.5.

FIG. 6. Reconstruction of the urinogenital system of the normal female of 32 mm. crown-rump length. × 9.5.

by Lillie ('23) for his 3.75 cm. one (Fig. 5) externally as well as in gross internal dissection. So it is not figured here in these aspects. The urogenital aperture is open although in the male of the same size and in the male twin it is closed and has a raphe

to mark the midline in place of a slit. The perineum is not expanded ventrally as in the male.

The vulva, vestibule, and bulbo-vestibular glands are female in type and proportions (Figs. 5 and 6). The ureters also come off the urethra or urogenital sinus as in the female from a ridge in the dorsal wall.

The wolffian ducts are not much affected though their lumen is greater just posterior to the mesonephros than in the control. It is not to be expected that they would be affected greatly since they are still the ducts of the functional mesonephros.

The müllerian ducts are not so large in section as the control and lack a lumen in some regions where there is one in the control. They appear to be intermediate between the male and the female and, as in the male, show some signs of regression. Their staining reaction is like that of the male (Figs. 1, 2, 3, 4). This difference in size is most apparent in the region just posterior to the mesone-phros where the wolffian ducts are larger. The slight difference in the type of entry into the vestibule shown in Figs. 5 and 6 is probably not significant as such differences are found in single females. The ostia tubæ abdominale at the anterior end of the müllerian ducts open out flat with lips on the ventral aspect of the anterior end of the mesonephros and show a tendency to be associated with the rete there. This rete appears to lead over into the mesonephros and to join a glomerulus as in the normals of both sexes at this age.

*The Gonads.*—Figs. 1 and 2 are from sections through near the middle of the right gonads of 32 mm. female and male respectively, and figures 5 and 6 are similar sections of left gonads, etc., of the same sized freemartin and female, drawn with camera lucida to the same scale. As stated above, these thick sections (25 and 30 micra) show up the regions of the gonad better than thin sections, and cortical regions stand out better. The gonads of the free-martin have the same relations to the mesonephros as those of the female. Rete ovarii are similar in that they do not extend into the middle of the gonad as they do in the male, but are found only near the mesenterial border of the gland (Willier, '21; Bissonnette, '24). The type of tunica albuginea is female but the medulla of the ovary does not show signs of as great activity

as in the control.    A cortex is present outside the tunica albuginea
and is continuous with the germinal epithelium or serosa coat
from which it appears to develop, though organization into defi-
nite sexcords is not evident in the preparations of either female or
freemartin.    The secondary sexcord region is not quite so thick as
in the control nor is the gonad quite so large in cross-section.    It
is about 21/23 of the normal in diameter.    The length of the
freemartin gonad is 1,740 micra while that of the control is 2,150.
This may or may not be within the limits of normal variation at
this age for no series of measurements of normal ovaries has been
made at this stage to determine these limits.    The difference in
size is apparent from the sections drawn to the same scale.
This would be even greater were both sections taken from exactly
the same relative level of the gonad.    The freemartin was cut
62/100 of the length from the front end and the female 58/100 of
the corresponding distance.    As shown in the reconstructions,
the gonads are larger in diameter posterior to their middle regions
but near them.    The blood supply of the freemartin and control
gonads is distributed through the tunica albuginea inside the
cortex, while, in the corresponding male, since there is no cortex
outside the tunica, the blood supply is superficial in the definitive
tunica albuginea, present at this stage.    In the testis at this stage
there is marked cellular growth in the medulla or primary sexcord
region; in the ovary this activity is less marked; and even less so
in the freemartin gonad.

The anlagen of the gubernacula or round ligaments are similar
to those of both sexes at this stage.    No sex differences in this
organ appear till later stages as described by Bissonnette ('24).

## Discussion.

Lillie ('16 and '17) and Keller and Tandler ('16) came to the
conclusion that the freemartin is a modified female, that sex-
determination as a female was consummated at fertilization, and
that the abnormalities are due to the action of a male hormone
passed to her through the vascular anastomosis in the united
placental circulations.    They believed that this was a case of
normal zygotic sex-determination followed by abnormal sex-
differentiation in the female twin.    This material supports such

a theory by showing a case near the initiation of the differentia-
tion process where the embryo is essentially female in type and
condition of the organs, but is slightly underdeveloped in respect
to the secondary sexcord region and müllerian ducts. This case,
taken in connection with other described cases, particularly where
the placentæ were in contact but not united by either capillaries
or arteries or veins, as described by Lillie ('17) and Keller and
Tandler ('16) and verified by the writer, appears to show that a
blood-carried agent or hormone is at work here and not some
agent like the "organizer" which Spemann ('25) shows so un-
questionably is operative in the early development of the am-
phibian larva. If such an agent were operating here, mere
tissue cohesion or connection would be sufficient to condition full
effect, and the great differences between freemartins of the same
embryonic age would be difficult to account for except on the
basis of variations in zygotic sex-balance of the two individuals of
the twins or differences in time of contact of the chorions. Now,
in cattle, mere contact is usually attained early by reason of the
great elongation of the trophoblastic vesicle; but vascular con-
nection is not always so early, and the effect appears to depend
on blood transfusion rather than on tissue continuity.

However, this case and others such as the one described by
Keller and Tandler ('16), in which there was slight vascular inter-
change accompanied by a scarlike welt across the place of junc-
tion of the chorions, reducing what they believed was once a
greater vascular connection to proportions too small to account
for the degree of modification, appear to show that even a capil-
lary anastomosis or an anastomosis of very small bloodvessels is
sufficient to condition the modification. The state of the organs
appears to depend on the time elapsed since the effective thresh-
old of hormone action is reached, rather than on a varying
amount of effective hormone above such threshold. This free-
martin and the 9.0 cm. one of Bissonnette ('24) both showed a
minimum vascular connection accompanied by a type of modifi-
cation, or degree of it, which suggests a marked development as a
normal female, probably for some time before the hormone be-
came active, followed by regression of the peculiarly female
organs and a delayed acceleration of the growth of the essentially

male parts. They do not support the assumption that there has been a slower than normal development of the female structures with a slightly faster development of the male ones over the whole period of union, as will appear from what follows.

Lillie's Fig. 11, page 68, of a gonad section from a 3.75 cm. freemartin, in which he found demonstrable vessels connecting the circulations of the twins with no clearly distinguishable place of chorionic union, shows a germinal epithelium together with three or even four layers of cells outside the tunica albuginea in some regions. These cells of the extra layers are irregularly arranged and not in definite cords. These extra cell layers over and above the single layer of cœlomic epithelium homologous to that in the bull of similar size (Fig. 12) he does not interpret as a sexcord region and cords are not possible with so few cells in any case. After inspection of the 32 mm. freemartin in this study and all the freemartins younger than the 9.0 cm. stages examined by the writer microscopically, all of which show the secondary sexcord region, it appears that these extra cells in Lillie's specimen may well be secondary sexcord primordia in which cords have not yet organized. Brambell ('27) finds that in the mouse cords do not organize till later and that in males the germinal epithelium becomes several cell-layers thick after the tunica albuginea forms but later thins out to a single layer. This latter situation does not develop in males in cattle as seen in Fig. 2 of this paper and Fig. 12 of Lillie ('23). Chapin ('17) in some of her gonad sections shows regions which she does not interpret as derivatives from secondary sexcord anlagen, but which appear in the light of this more recent younger material and from an inspection of her slides themselves (kindly loaned by Professor F. R. Lillie) to be derived from such source with possible later proliferation. In any case the secondary sexcord region is not a layer of uniform thickness all over the gonad but is thicker in lateral regions than in ventral. The layer also appears to be resorbed at different rates in different regions and so to remain in some regions longer than in others and possibly permanently. This appears to be correlated with or dependent on pressure by adjacent organs in some way. It is possible for the tunica albuginea to reach the outside of the gonad in some regions and not in others, leaving bands of secondary

sexcord derivatives extending lengthwise in the gonad parallel to the medullary cord derivatives. This situation is figured by other workers. These remnants were difficult or impossible to interpret without comparison with the earlier stages or with stages which had undoubted secondary sexcords. Such a series was not available for study by other students of the freemartin. This will be discussed more fully in a later paper on the basis of more material.

From a study of the above mentioned freemartins and of a 6.75 cm. and 7.5 cm. pair from a set of triplets with one bull only (to be discussed in a later paper) where cords of Pflüger are also present but show signs of regression, one is led to conclude that the rate of resorption of these cords is comparatively slow as well as that they may continue to grow in some cases for a short time before regressing. This also leads one to believe that secondary sexcord regions were never present in Lillie's specimen mentioned above, in any greater amount than is shown in his figure (Lillie, '23, Fig. 11, p. 68). This also suggests that the initiation of the male hormone action as inhibitor of the secondary sexcord region was longer delayed in this 32 mm. one than in Lillie's 3.75 cm. one. This may be explained on the basis of the small amount of hormone passing to the female at first through the small vascular connection. This would delay the effective minimum concentration of the hormone. That there is such an effective minimum, in some mammals at least, is shown by the partial castration and grafting experiments of Moore ('21), Sand ('19), Steinach ('20), and many others for postnatal stages. No prenatal testis grafts have yet been possible in mammals so far as the writer can learn.

It is possible that the vascular anastomosis was just becoming established in these twins and that, as the pregnancy progressed, it would have become more effective by the pressing together of the chorions. This would insure a continuous larger supply of hormone on the female side and the rate of modification in this case would be as fast as in those with earlier complete anastomosis, though the condition reached at any time would lag behind that of the others and this freemartin would fall in the group showing small degree of modification as classified by Willier ('21).

Willier ('21) classifies freemartins into three classes on the

basis of degree of modification—those with small, medium, and large amount of abnormality—and Lillie states that the freemartins "form an exceedingly well-defined group without intergrades to normal females or normal males." This may be true for postnatal freemartins; but, from the series discussed in this and other papers cited, it appears that a series of intergrades connects the female with the freemartin group, or that the freemartin group moves steadily away from the female group of types, as we pass from younger to older stages. No such series is seen between the freemartin and male. The rare cases of modification externally in the male direction are probably due to upset of some kind in genetic or zygotic sex balance and not to the freemartin type of hormonic modification alone. Such cases occur without twinning in cattle, and in as great a percentage of cases as in freemartins. It is this series in the younger stages leading back to the normal female type among freemartins that so conclusively shows that the freemartin is a modified female and not a modified male. Taken with Lillie's ('17) statistics as to sex-ratios in cattle twins, it furnishes most conclusive proof of the theory of the freemartin of Lillie and of Keller and Tandler.

Lillie ('23) suggests that probably there are individual differences in zygotic balance of sex factors in cattle even in single born calves, and those who have been much associated with cattle breeding and judging, and have seen the differences in potency of males and females inter se, and their differing degrees of male and female "conformation," will agree. This appears to be a fundamental factor in the condition of all freemartins and doubtless works from both sides, male as well as female, in the pair. The hormone, even, may not be equally potent in all cases. Its appearance may be delayed in some males for this reason. So the initial action may be delayed even in cases with early complete intercommunication of blood vessels. The females also may differ in their resistance to the masculinizing action of the male hormone: they may differ already in femaleness. Comparison between this case and Lillie's and other small freemartins illustrates how these factors may lead to variations, among the early stages at least. It is also just possible that the differences from the control female found in this specimen may be

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within the limits of individual variation resulting from these differences in zygotic sex balance. Only a careful study by biometric methods of a large number of single female embryos at this stage can settle this point. Such a study has not been made because of lack of material and the long time involved in search.

In any case, should this case fall inside the limits of individual variation discussed in the preceding paragraph, it will show all the more conclusively that the first stage of the freemartin is, at least, in some cases, a female, normal for that stage of development.

## CONCLUSIONS.

1. On the basis of this 32 mm. freemartin and comparison with others described by the writer and by others, where the freemartin modifications are conditioned by vascular unions between the placentæ and not by mere contact and adhesion of one to the other, it is concluded that a blood-carried agent in the nature of a hormone is effective in producing the freemartin rather than an "organizer" of the type found in Spemann's work on early amphibian development.

2. Small vascular or capillary anastomosis is sufficient to condition the freemartin effects in some cases at least; but such small amounts of transfusion as are possible in such cases at first appear to delay the modifications and permit longer female development before the effective minimal concentration of hormone is reached on the female side. Early stages in such cases show a more markedly female embryo than do those with strong anastomosis, particularly in respect to gonad and müllerian ducts. Study of such borderline cases as these lend support to the "all-or-none" conception of the hormone action.

3. Cords of Pflüger or secondary sexcord regions develop frequently in freemartins and are resorbed later at unequal rates in different parts of the gonad, so that some of the peculiarly complicated interiors of gonads figured by previous students of the freemartin gonad can be interpreted in the light of these earlier stages as the results of survivals of isolated parts of the secondary sexcord region, separated from the medulla of the gland by tunica albuginea. Organization into sexcords may not be possible from the first and some individuals may not develop

cords though they may have the cell proliferations from which the cords would later develop but for the action of the hormone. This unequal development of sexcord region and unequal resorption seem to be related in some way to pressure by surrounding organs.

4. There is no intergrading in freemartins between the freemartin types and male types except in rare cases which resemble the anomalies found among single born males, where the sex balance appears to be upset. And in postnatal freemartins there is no intergrading to the female type. But in prenatal freemartins there is a definite series of intergrades to the female condition as the younger stages are studied till practically the female condition is reached. This furnishes the strongest of proof for the theory of the freemartin maintained by Lillie and by Keller and Tandler.

5. There are probably individual differences in zygotic sex balance among both male and female cattle. This is a factor in the variations of condition among freemartins and doubtless is operative from the bull's side as well as on the freemartin side. The hormone may not be equally potent in all cases and it may possibly differ in potency at different times in the same male, or be delayed in its appearance. In this way the hormone action may be delayed even when early complete anastomosis is effected. So, too, it is possible that the differences between this freemartin and the control female may be within the limits of individual variation correlated with zygotic differences in sex balance, for the exact limits of such variation have not yet been determined.

6. If so, it is even stronger proof for the theory of the freemartin of Lillie and of Keller and Tandler, since it would show that the first stage of the freemartin is a female, normal in sexual condition for that age.

## SUMMARY.

1. A 32 mm. freemartin twin to a 34 mm. male is compared with a normal female of like size as to reproductive organs, and with normal male as to gonad.

2. It differs from the female control in having a thinner secondary sexcord region and thinner müllerian duct, lacking lumen in places where the control has one. Nevertheless it is more like

the female than any other freemartins with vascular anastomosis so far studied.

3. Placental vascular anastomosis between the twins was very slight—hardly more than capillary—and the placentæ pulled apart with slight bleeding. So it permitted very limited transfusion of male hormone, a situation correlated with slight modification from female type shown in small gonad and thin ovarian cortex and müllerian duct.

4. Comparison with other freemartins as to placental and sexual conditions indicates a blood-borne hormone as the agent rather than an "organizer" found effective in early amphibian differentiation. Mere tissue cohesion fails to condition the modifications as it does in Spemann's transplants.

5. Comparison with other gonads figured for freemartins and with new material indicates that some formerly anomalous regions in older freemartin gonads are derived from secondary sexcord remnants which failed to be resorbed and that these cortical regions frequently develop in freemartins to some degree and may or may not disappear.

6. This study adds support to the theory of the freemartin of Lillie and Keller and Tandler, that it is female, normally determined zygotically, but abnormally differentiated sexually by the intervention of a male hormone received from her twin brother in utero.

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## ABBREVIATIONS.

*Bl.*, bladder.

*B. V. Gl.*, bulbovestibular or Bartholin's glands.

*Clit.*, clitoris.

*Gon.*, gonad.

*Gr. Ph. Un.*, undercut groove in phallus.

*Gub.*, gubernaculum.

*Mesoneph.*, mesonephros.

*Mül. Dt.*, müllerian duct.

*Ost.*, ostium tubæ abdominale.

*Rect.*, rectum.

*Sac. Vag.*, saccus vaginalis.

*Scr.*, scrotum.

*Ura.*, urethra.

*Vest.*, vestibule.

*Vul.*, vulva.

*W. Dt.*, Wolffian duct.